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(56) Documents cited
J. Virol. 1981, 38(2), 688-703
Gene 1984, 32, 389-398

(58) Field of search
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Online databases: WPI, DIALOG/BIOTECH

(54) Retroviral vector and its use in the production of transgenic animals

(57) Retroviral vectors carrying foreign nucleic acid and having a Feline Leukaemia Virus B *env* gene product can be introduced into the embryos of sheep and pigs. The foreign nucleic acid is incorporated into the embryo chromosomes, producing transgenic animals. Introduction of a retroviral vector into any embryo is improved by injecting it through the zona pellucida into the perivitelline space.

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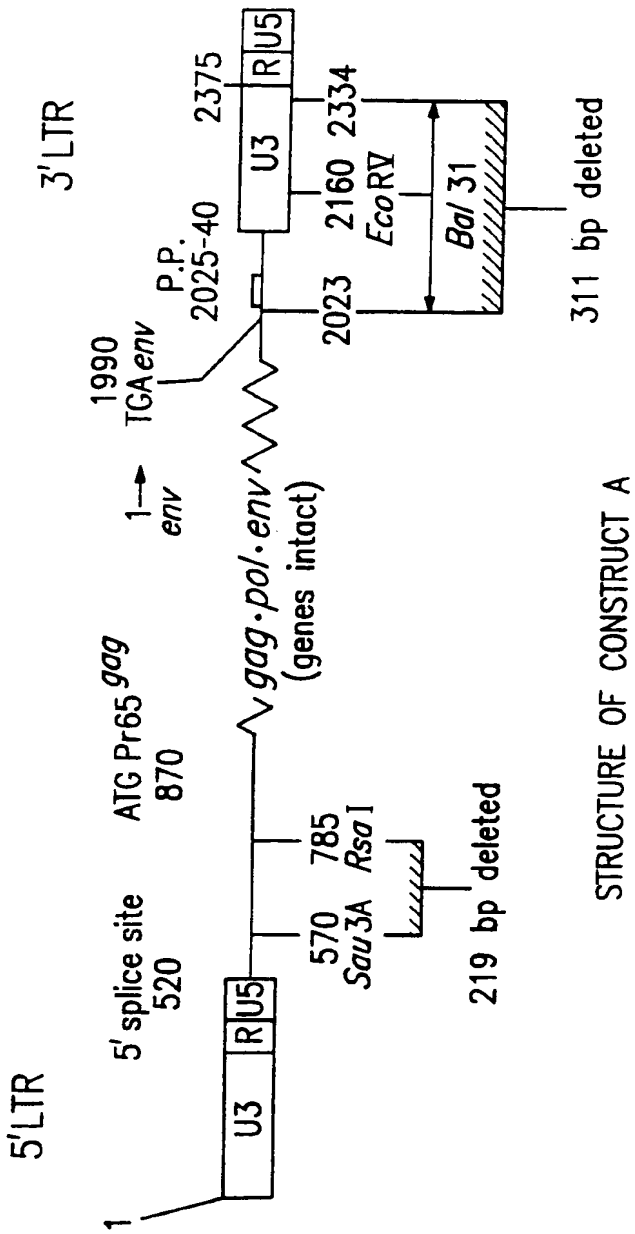
Fig. 1



STRUCTURE OF CONSTRUCT A

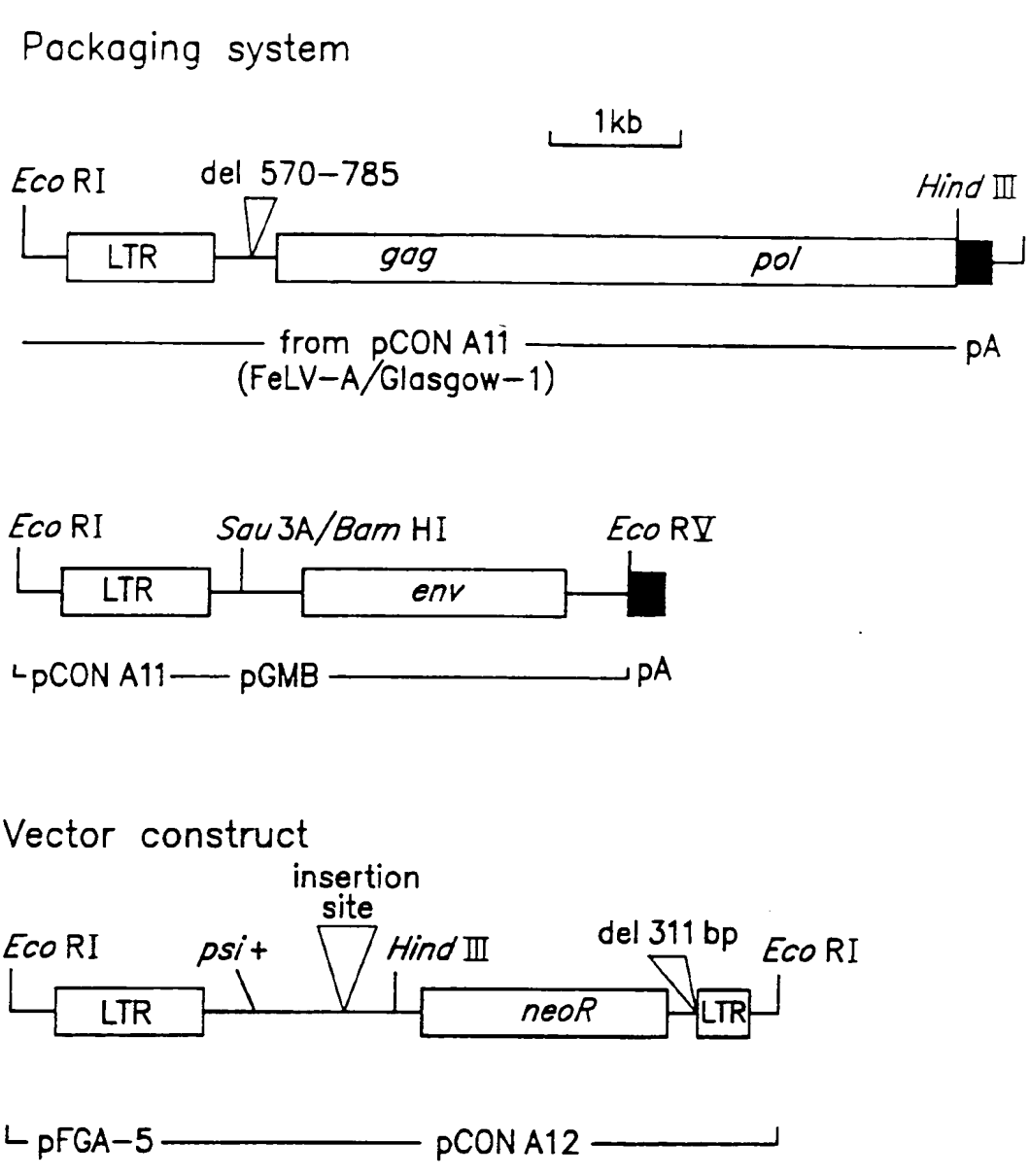
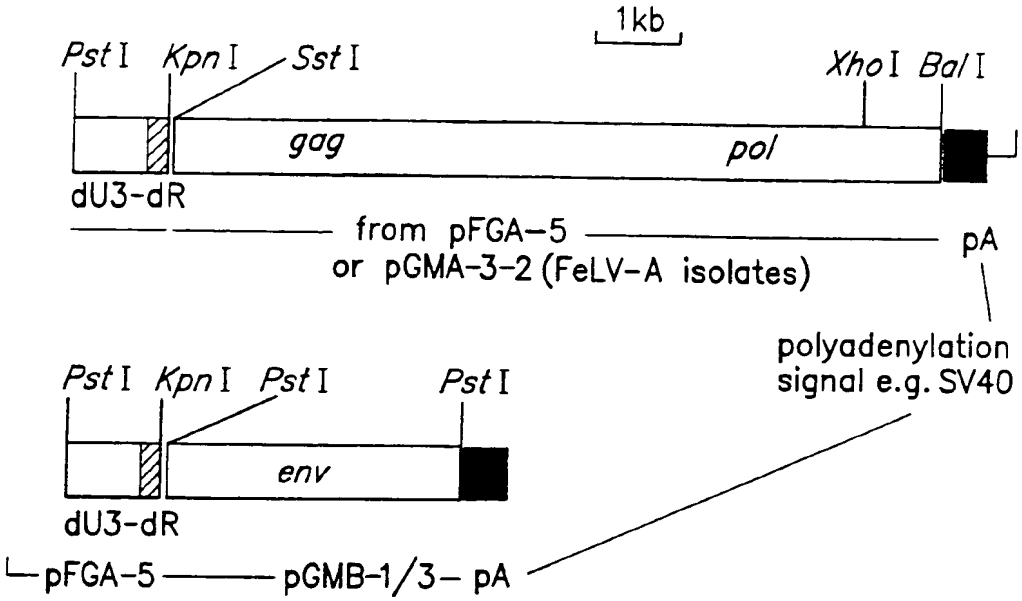

Packaging system
1kb
Eco RI
del 570–785
Hind III
LTR
gag
pol
from pCON A11
(FeLV–A/Glasgow–1)
pA
Eco RI
Sau 3A/Bam HI
Eco RV
LTR
env
pCON A11
pGMB
pA
Vector construct
insertion site
Eco RI
psi+
Hind III
del 311 bp
Eco RI
LTR
neoR
LTR
pFGA–5
pCON A12

Fig. 2

Packaging system



Vector construct

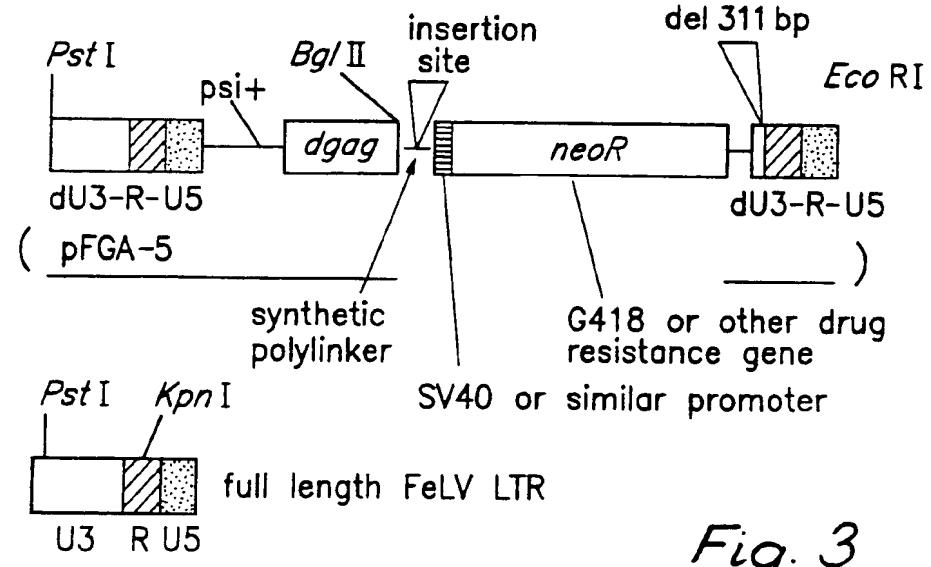


Fig. 3

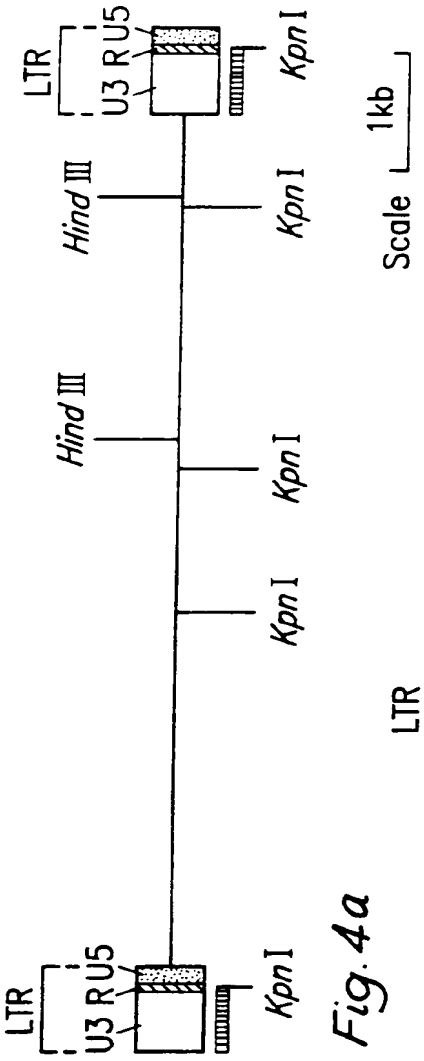

LTR
U3 R U5
KpnI
HindIII
KpnI
HindIII
KpnI
KpnI
KpnI
LTR
U3 R U5
KpnI
Scale 1kb

Fig. 4a

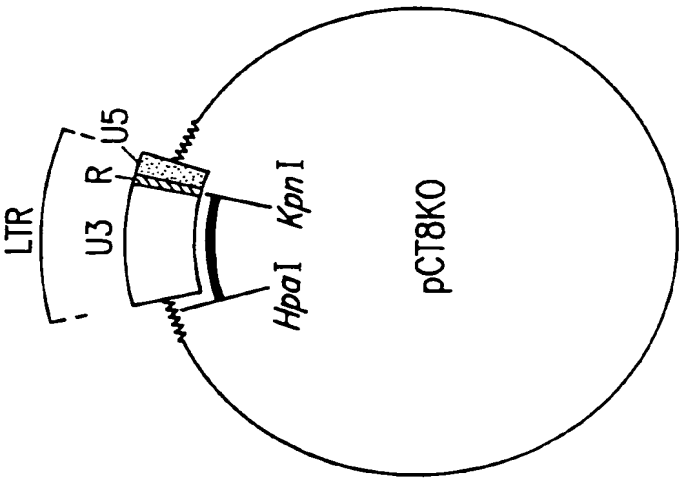

LTR
U3 R U5
KpnI
HpaI
pCT8KO

Fig. 4b

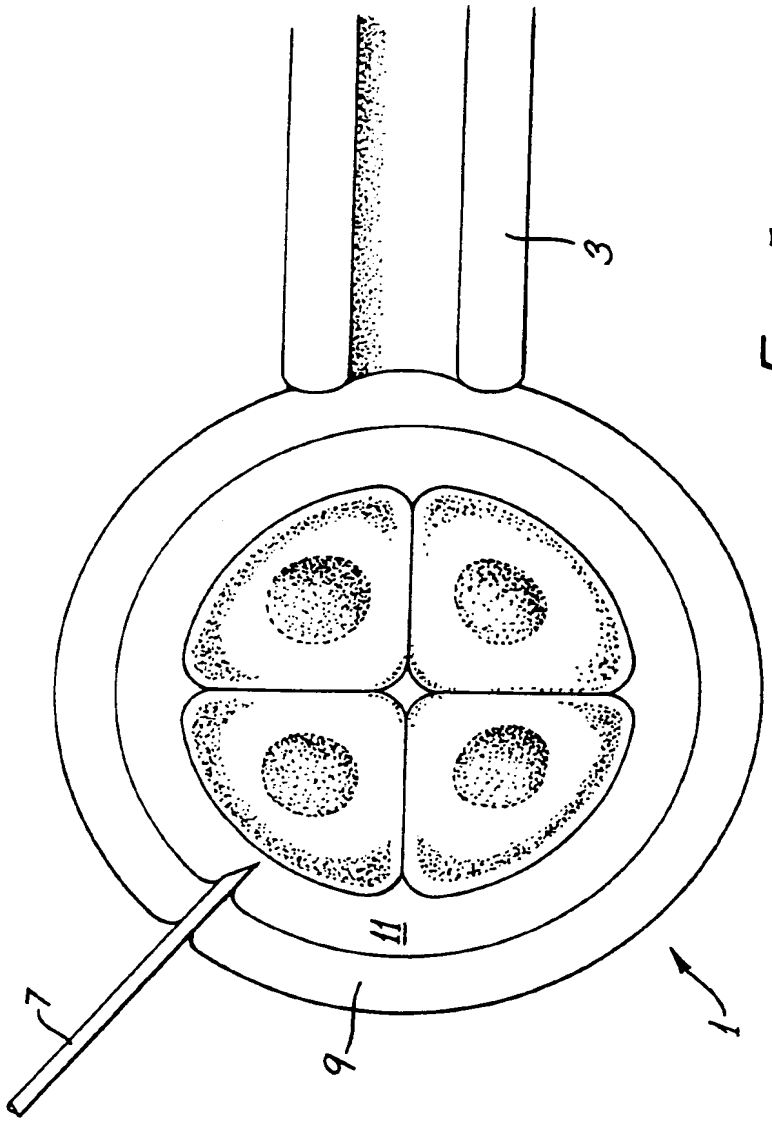


Fig. 5

RETROVIRAL VECTOR AND ITS USE IN THE PRODUCTION OF TRANSGENIC ANIMALS

Background of the invention

1. Field of the invention

This invention relates to a retroviral vector and its use in the production of transgenic animals, particularly domestic livestock animals such as sheep.

2. Description of the prior art

Transgenic animals are those in which exogenous DNA has been introduced into the cells of an embryo such that the introduced genetic information is present in many cells of the adult. In the case of germ line transgenics the introduced DNA is passed onto succeeding generations through the normal reproductive processes. Provided appropriate transcriptional control elements have been incorporated, introduced genes may be expressed in the tissues of the transgenic animals.

The production of transgenic animals, such as domestic animals including sheep, has a number of commercial applications. These applications include the production of animals resistant to specific infections or the production of animals with enhanced growth rate or reproductive performance. In addition, the expression of exogenous proteins in specific tissues compartments of sheep provides a means of harvesting commercial quantities of these proteins.

A limiting factor in the production of transgenic animals is the efficiency with which the primary transgenic animal can be produced. The most widely used method of producing both transgenic mice and sheep is by pronuclear injection of a newly fertilised ovum. While this procedure is efficient in mice it is much less successful in sheep.

An alternative method of producing transgenic animals is to infect early embryos with retroviruses or vectors derived from retroviruses. In mice transgenic animals have been produced by infection of early embryos (Jaenisch, et al., 1976: Van der Putten et al., 1985; Jaenisch et al., 1981). However, these procedures involve removal of the zona pellucida and the plating

of the naked embryos over cells, releasing retroviruses and retrovirus vectors (van der Putten et al., 1985). Removal of the zona pellucida from ovine embryos results in a significant decrease in the viability of the embryo. This problem can be overcome by a procedure described hereinafter.

Although there have been concerns about the safety of retroviruses, certain vectors known as self-inactivating vectors have been produced from murine retroviruses (Yu et al. 1986). These vectors are designed so that the risk of activation of cellular oncogenes or of undesired transcription of wild type retroviral genes is negligibly low.

However, a factor limiting the use of retrovirus vectors in domestic animals has been the choice of an appropriate retrovirus since not all retroviruses are capable of infecting animal embryos at an early stage of embryonic development.

Summary of the invention

It has now been found that retroviral vectors containing the env gene product of feline leukaemia virus sub-group B can infect the embryos of sheep and pigs, and that the virus, or components of the virus, can be used in the construction of a vector for preparing transgenic animals.

A retroviral vector is usually defined as comprising both the genetic material and the proteinaceous structure, which acts as the vehicle for introducing the genetic material into cells. Therefore, according to a first aspect of the invention, there is provided a retroviral vector comprising (1) gag and pol gene products of a retrovirus; (2) the env gene product of feline leukaemia virus sub-group B (FeLV-B) and (3) retroviral sequences required for reverse transcription of retroviral RNA into DNA, for packaging, and, where integration of the transcribed DNA into the chromosome of a cell is required, also for integration, said retroviral sequences further containing foreign nucleic acid. Included within this aspect are cell cultures, especially cell lines, containing particles of the retroviral vector or capable of releasing them upon introduction into the animal embryo.

The foreign nucleic acid will code for, or be complementary to nucleic acid coding for a gene product of interest, and will therefore be different from natural FeLV-B genes.

The retroviral vector may contain the gag and pol gene products of FeLV-B, in addition to the env gene product, but gag and pol gene products of other subgroups of feline leukaemia virus, such as of the A subgroup or indeed of any oncovirus of the family retroviridae, may be present.

Although it will usually be the natural env gene product from FeLV-B that is present in the vector, there is in principle no reason why a functionally equivalent mutant, recombinant or synthetic protein could not be substituted. The function of the env gene product is to permit binding to, and entry of the vector into, the target cell.

The nucleic acid will, at the present state of technology, generally be packaged with the proteinaceous components as RNA, although developments which enable DNA to be packaged in infectious retrovirus virions are not excluded from the scope of the invention.

The general principles of retrovirus vectors have been described (Mann et al., 1983; Miller et al., 1986; and Eglitis and Anderson, 1988). Such disclosures are herein incorporated by reference. The vector is normally produced from a packaging cell line. The foreign nucleic acid to be incorporated in the vector is introduced into the packaging cell line, e.g. by DNA transfection or by retroviral infection. The packaging cell line contains one or more nucleic acid molecules collectively providing gag, pol and env genes, but lacks a packaging function, with the result that it produces only empty virus particles which lack internal virion RNA.

According to a second aspect of the invention, there is provided a packaging cell line containing nucleic acid coding for retroviridae packaging proteins, wherein the env gene product is of FeLV-B, i.e. is derived from or functionally equivalent to that of FeLV-B.

The packaging cell line may contain one or more nucleic acid constructs (often proviral DNA constructs) comprising genes coding for all the viral proteins necessary to produce an infectious virion. If all the proteins are derived from FeLV-B, then only one packaging cell line construct need be used. If proteins other than the FeLV-B *env* gene product are derived from other retroviruses, then it may be convenient to transfect a starting cell line with separate constructs containing *gag-pol* genes and the FeLV-B *env* gene, respectively. The construct(s) will generally be so adapted or configured that the RNA transcripts will not be packaged into virions. This can be achieved by, for example, making one or more effective modifications to the packaging sequence of the retroviral nucleic acid. While these modifications are usually deletions, they can also be substitutions, whether by point mutations or longer sequences.

Packaging cell lines in accordance with the second aspect of the invention are useful intermediates in the production of vectors in accordance with the first aspect. Foreign nucleic acid, which may comprise or correspond to a gene of interest can be introduced, e.g. by transfection, into the packaging cell line as a 'vector construct', which is so called to be distinguished from the packaging cell line constructs described above. The vector construct will also generally contain genetic information in the form of a packaging sequence to ensure that RNA corresponding to the foreign DNA is packaged within the viral protein assembly.

It is possible to construct a self-inactivating (SIN) vector from feline leukaemia virus; this may have one or more deletions within the long terminal repeats (LTRs). Transcription is not initiated from proviral DNA produced from self-inactivating (SIN) vector constructs having deletions of promoter and enhancer within the 3'-long terminal repeat (Yu *et al.*, 1986). Such deletions give rise to progeny proviral DNA (in the target cell) having the deletions in both 5'- and 3'-LTRs, and without the

5'-LTR promoter and enhancer transcription of the progeny proviral DNA into further viral RNA cannot take place. Consequently such SIN proviruses cannot be rescued by wild type virus, nor can they initiate the most widely recognised mechanisms of leukaemogenesis associated with the class of virus.

According to a third aspect of the invention, there is provided a process for the production of a transgenic animal, the process comprising introducing a retroviral vector in accordance with the first aspect, or a retroviral nucleoprotein complex containing the DNA of said retroviral vector, intracellularly into an appropriate animal embryo.

The vector may be introduced intracellularly by means of injection. In a preferred aspect, the process involves microinjection of the vector through the zona pellucida into the subzonal (perivitelline) space. It would also be possible to inject cells releasing retroviruses or other retrovirus vectors into this space. Both procedures retain the zona pellucida intact, thereby favouring the survival of the manipulated embryo.

The animal may be a sheep, but the invention may have application in the production of other transgenic animals, particularly large domestic mammals such as pigs, whose embryos are difficult to manipulate by conventional techniques.

According to a fourth aspect of the invention, there is provided a transgenic animal produced by means of a vector, cell line and/or process as described above.

The invention also encompasses an improved process of introducing nucleic acid into an animal embryo, the process comprising injecting a viral vector or vector-releasing cell line through the zona pellucida into the subzonal (perivitelline space). The nucleic acid will usually be in the form of a retroviral vector, such as a retroviral vector in accordance with the first aspect. This is an improvement on the prior method of removing the zone pellucida.

Brief description of the drawings

Figure 1 shows the structure of a molecularly cloned feline leukaemia virus of subgroup-A;

Figure 2 shows constructs used to produce a packaging cell line when transfected into feline cells; and a SIN vector construct for introduction into the cell line.

Figure 3 is analogous to Figure 2 but shows different constructs for both the packaging cell line and the SIN vector;

Figure 4a is a diagram of the Sarma-B FeLV-B provirus used in the Examples;

Figure 4b is a diagram of the plasmid pCT8KO from which a probe fragment was isolated; and

Figure 5 shows schematically how vectors may be injected through the zona pellucida.

Description of the preferred embodiments

This invention relates in a preferred aspect to an improved method for the production of transgenic sheep. The method utilises retroviruses and vectors derived from retroviruses to introduce and integrate genes into the chromosomal DNA of early embryos.

In preferred embodiments, a concentrated suspension of feline leukaemia virus of subgroup-B and/or vectors derived from this retrovirus are concentrated to from 1 to 10 x 10^6, preferably about 5 x 10^6 ffu/ml (focus forming units per millilitre) and injected through the zona pellucida into the perivitteline (intrazonal) space of 2-8 cell stage embryos. A volume sufficient to cause visible swelling of the periviteline space may be microinjected. Embryos manipulated in this way can be produced by standard multiple ovulation techniques described below and transferred to synchronised recipient ewes and allowed to develop to term.

The invention in its various aspects is an improvement on conventional techniques in three principal respects. First it is approximately 10-fold more efficient at producing transgenic sheep than conventional pronuclear microinjection techniques. Efficiency is defined here as the number of transgenics produced at 50 days of gestation or at birth compared to the number of embryos manipulated. Table 1 lists the efficiency of transgenic

sheep production using pronuclear microinjection compared to the use of the retrovirus FeLV-B.

Table 1

Method	Number of Embryos	Foetuses surviving	Number (% transgenics) of embryos manipulated
Pronuclear injection	2745	550	13 (0.47)
Refs: Hammer et al. (1987) Nancarrow et al. (1987) Pursel et al. (1988)			
Retrovirus FeLV-B	47	23	2 (4.2)

Secondly, the method utilises the natural infective processes of the retrovirus. During infection the genome of the retrovirus or vector is transcribed into DNA by the virion reverse transcriptase and integrated into chromosomal DNA through the integrase function of the pol gene product. This retroviral integrative procedure results in a low copy number of unrearranged proviruses, whereas microinjection often results in integration of mutliple genes in complex arrangements or concatamers.

The integration of a low copy number of unrearranged genes has the advantage that animals may be more easily bred to homozygosity with respect to the introduced gene. It is believed that this will be a requirement in the United Kingdom for release of animals from Home Office Licence into commercial use.

Thirdly, this method is technically simpler than pronuclear microinjection. Pronuclear microinjection is difficult in ovine embryos and embryos of other domestic livestock as the pronuclei are often obscured by yolk material. Although the pronuclei may be visualised by centrifuging the embryos to sediment the yolk, injection of the pronuclei is both an invasive technique and requires a high degree of operator skill. In contrast, preferred aspects of the present invention only require that a micropipette be inserted beneath the zona pellucida which is technically simple.

This invention may be used for the efficient introduction and integration of DNA sequences into the chromosomal DNA of embryos of appropriate animals, as explained above, especially sheep. This invention will be useful for, but not limited to, the following:

(1) the introduction of genes that modify growth rate growth size or carcass composition (for instance genes like a natural or other growth hormone);

(2) the introduction of genes that confer disease resistance to viruses or other microorganisms (for instance, for intra-pathogenic microorganisms these could be

(i) genes that block the microorganisms receptor on the cell surface,

(ii) DNA sequences that are transcribed into anti-sense RNA of an essential gene of the micro-organism or

(iii) DNA that is transcribed into RNA sequences that catalytically cleave specific RNAs (Ribozymes) - ribozymes can be designed to cleave specific targets like viral RNAs, thereby conferring resistance to virus infection; or

(3) the introduction of genes of a foreign protein of commercial value that is expressed in, and harvested from, a particular tissue component.

Embodiments of the invention will now be described by reference to the Examples and the accompanying drawings.

Exemplary molecular clones and DNA sequences for producing an FeLV-B packaging line and SIN vector system are as follows:

(1) Figure 1 shows the structure of a molecularly cloned feline leukaemia virus of subgroup-A. Deletion of the sequences 570-785 lying between the restriction endonuclease sites Sau3A and RsaI results in deletion of a 219 bp packaging sequence (psi) necessary for incorporation of genomic RNA into virions.

(2) A packaging cell line can be produced from two constructs transfected into feline cells. The first element shown in Figure 2 consists of molecularly cloned FeLV-A/Glasgow-1 with a deleted packaging sequence. This construct is designated pCON A11. The

5' long terminal repeat (LTR) together with the gag and pol genes from pCON A11 form the first packaging construct.

The second packaging construct consists of the LTR from pCON A11 ligated to the envelope gene (env) of molecularly cloned FeLV-B. These two constructs, when transfected into a cell line, provide a packaging cell line having all the necessary vital functions to package viral or vector construct RNA into a subgroup-B envelope.

(3) A SIN vector with a selectable marker gene neoR, and an insertion site for a foreign gene or genes of interest can be constructed as follows. Deletions in the U3 region of the 3' LTR of FeLV-A/Glasgow-1 remove the enhancer and promoter sequence while sparing the terminal redundancy (R) region. This is construct pCON A12.

Ligation of this element to a functional LTR and a downstream packaging sequence in construct pFGA-5 produces a SIN vector construct. Transfecting this SIN vector construct into the packaging cell line above would result in incorporation of SIN vector genomic RNA into infectious particles having a FeLV subgroup-B envelope. These vectors would be capable of infecting ovine embryos and of introducing any gene of interest incorporated into the vector.

(4) A second example of packaging system constructs useful for making a packaging cell line is shown in Figure 3. The constructs encoding gag-pol and env genes are more substantially deleted in non-coding sequences. These modifications are intended to reduce the probability of recombinations which could generate wild-type FeLV. The gag-pol and env genes in this second example are still expressed from the FeLV LTR promoter. However, sequences 5' to a conserved LTR PstI restriction site are removed, as are sequences 3' to a conserved KpnI site. The gag-pol fragment extends from an SstI site 5' to the Pr65gag initiation site and ends at a BalI site at the initiator codon for the env gene product. The env gene-expressing construct utilises conserved PstI sites 5' of the env gene and 3' to env,

within the LTR. The locations of these restriction sites can be found in Stewart et al., 1986.

(5) In Figure 3, a second self-inactivating vector construct example is shown whereby a portion of the gag coding sequence is included (up to a conserved BglII site, see Stewart et al., 1986) to maximise packaging of the vector construct RNA into particles. Also, the drug resistance marker is expressed from an internal promoter. The foreign gene of interest could be introduced into such a construct in either orientation and with its own promoter.

The various starting materials referred to may readily be constructed by those skilled in the art, and in addition molecular clones of feline leukaemia virus such as pFGA-5 and pGMB are available for research purposes from The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD.

The following Examples demonstrate successful injection of wild type FeLV-B into sheep embryos. Since it is the interaction between receptors or other proteins on embryo cells and the env gene products of FeLV-B which is responsible for the success of this invention, it follows that FeLV-B packaged with foreign nucleic acid will be similarly successful in infecting sheep embryos.

Example 1

Production of Virus

Feline leukaemia virus of subgroup-B was grown in the feline embryo cell line FEA. Persistently infected cells were plated at 1 x 10^6 in 9 cm tissue culture dishes overlaid with 5 ml of McCoy's 5A medium with 10% foetal bovine serum and 20 ug/ml gentamycin (complete medium). Three days later the medium was removed, clarified at 10,000g and stored at -70°C.

The virus was concentrated by ultracentrifugation of the stock virus at 21,000 rpm for 1 hour in a Beckman SW28 rotor. The pelleted virus was resuspended in complete medium and an aliquot taken for titration. The remaining virus was placed on

ice and used within 6 hours for microinjection. Virus was titrated by the FEA/c81 assay as previously described (Fischinger et al., 1974). The concentrated virus stock used fell within the range of 5×10^6 to 7.2×10^6 ffu/ml.

Example 2

Harvesting of Fertilised Eggs

Female sheep were synchronised using vaginal tampons containing progestagens (Veramix, Upjohn) which were left in the vagina for 14 days. At the time of sponge withdrawal, 1500iu Pregnant Mare's Serum Gonadotrophin (Folligen, Intervet) in 2 ml diluent (Phosphate Buffered Water) was given by intramuscular injection. The ewes were then allowed access to rams until mated, usually between 18 and 30 hours after sponge removal.

The fertilised eggs were removed from the sheep under general anaesthesia at between 76 and 84 hours after tampon removal. This procedure was conducted as follows. Anaesthetic induction was induced by intravenous Pentobarbitone at the rate of 25mg/Kg. Intubation was carried out with the aid of a laryngoscope using a suitable sized endotracheal tube and anaesthesia was maintained with 3% Halothane in Oxygen. Risks of ruminal contents being regurgitated and inhaled were eliminated by the placing of a 2cm diameter stomach tube into the oesophagus.

Once the sheep was fully anaesthetised, it was placed in dorsal recumbancy and supported in that position throughout the operation. The abdomen was shaved, cleaned and disinfected prior to surgical drapes being placed in position, leaving only the midline incision site exposed. A midline skin incision was made from just behind umbilicus posteriorly to just in front of the mammary gland. Blunt dissection was carried out to reveal the linea alba, through which was made an incision, of similar length to the skin incision. The uterus was identified, exteriorised and the opening to one of the uterine tubes identified by lifting the ovarian bursa from the ovary. A sterile glass tube of outer diameter 29mm and internal diameter 24mm was bent in a flame into

a 45° angle and the flame-rounded end was inserted about 2.5cm down the uterine tube from the ovarian bursa. A warm sterile embryo collecting dish (Camlab, Cambridge) covered with sterilised aluminium foil was placed under the open end of the collecting tube and this was pushed through the foil in order to maintain the sterility of the dish.

The uterine horn was grasped about 5 cm from the uterotubal junction and the lumen was occluded at this point by digital pressure. A sterile 20ml syringe was filled with 15ml of Ovum Culture Medium (OCM) (Flow Labs., Irvine) at 37°C and a 20g needle attached. The needle was inserted into the lumen of the horn between the occluded portion of the horn and the utero-tubal junction. The Medium was gently flushed into the uterus, up through the uterine tube, into the glass tubing and out into the collecting dish. The dish was immediately covered with a second layer of foil and put into a 37°C incubator. The other uterine tube was similarly flushed.

The peritoneal layer of the sheep was closed using an interrupted layer of 4 metric Nylon, a subcuticular layer of 3.5 metric catgut and the skin incision closed using 14 mm Michel clips. Antibiotics were routinely given, and the animals closely observed during and after anaesthetic recovery.

Embryo manipulations were carried out in a horizontal laminar flow hood wherever possible. Eggs were searched for under a stereo-dissecting microscope at a magnification of X 50 and were drawn up into a finely pulled glass pipette whose internal diameter was slightly larger than the egg diameter (approx. 200 mcMO. They were transferred to 35 mm petri dishes containing OCM plus 20 mM Hepes. Once all the eggs were found in the flushing fluid they were replaced in the incubator until micromanipulation could be carried out. This is normally within 1 hour of collection.

Example 3

Microinjection of Virus

The insertion of virus was achieved using 2 Micromanipulators (Leitz) and an Invert Microscope mounted on a common Baseplate (Leitz). The left micromanipulator instrument holder carries a finely drawn holding pipette, of an outer diameter of 100 mcM and an internal diameter of 30 mcM. This was connected by pressure tubing to a Leitz screw syringe and the system filled with Fluorinert 77 (Sigma). The right manipulator carried the injection pipette broken to a tip diameter approximately 3-5 microns which was connected by pressure tubing to a 100 ml glass syringe (Sigma, Italy). The injection pipette was drawn on a pipette puller (Campden Instruments, London) using glass capillary tubing with fine internal filament (Clark Electromedical Instruments GC100TF-15). A small amount of the virus in tissue culture medium was loaded into the pipette which was then filled with Fluorinert 77, before being placed in the instrument holder of the manipulator.

Figure 4a is a diagram of the Sarma-B FeLV-B provirus (viral DNA) used in this Example showing the positions of the KpnI and HindIII restriction sites. The hatched horizontal bars indicate those parts of the provirus homologous to the radiolabelled U3 probe DNA used. The long terminal repeats (LTRs) in this wild type virus are intact and it contains packaging sequence and gag, pol and env genes.

The eggs were placed into a chamber constructed from glass. Small pieces (5 x 25mm) were cut from microscope slides (75 x 25mm) and on each long side of a slide 3 such pieces were glued to make walls approximately 15 mm high. These were autoclaved at 134°C for 7 minutes and were re-used after laboratory standard cleaning. Sterile petroleum jelly was smeared on the top of the walls of the chambers and a sterile 22 mm x 22 mm coverslip was placed on top and embedded into the petroleum jelly.

Warm OCM including 20 mM Hepes was run under the coverslip until the chamber was filled. Suitable cleaved eggs were taken from the holding petri dish with a fine pipette and placed in the chamber.

Once the chamber was placed on the Invert Microscope, the holding pipette and the injecting pipettes were manipulated into the chamber and, as can be seen in Figure 5, one egg 1 was gently drawn onto the holding pipette 3 using gentle suction. The injection pipette 7 was then stabbed through the Zona Pellucida 9 avoiding the blastomeres and approximately 5 nanolitres injected into the intrazonal (perivitelline) space 11. The procedure was repeated for all the available eggs. These were then transferred back into an incubator in OCM plus 20 mM Hepes.

After about 1 hour, the injected eggs were examined microscopically for signs of lysis or damage and any such eggs were discarded. Using mouth suction through a length of fine plastic capillary tubing, 3 eggs were loaded into a sterile 10 cm long capillary tube (Clark GC100-10) one end of which was rounded by flaming in a micro-Bunsen burner.

Example 4

Transfer of Eggs to Recipients

The eggs were transferred into sheep which had been synchronised in a similar fashion to the donors, the only differences being that the recipients were given only 500 iu PMSG and that their sponges were removed about 12 hours before those of the donors. The surgery of the recipients was identical to that of the donors except that the eggs were transferred by inserting the pipette into one uterine tube and gently expelling them by blowing through the plastic tubing.

The sheep which remained pregnant with the transferred eggs were slaughtered at between 42 and 50 days, and their reproductive tracts recovered and taken back to the laboratory, where dissection took place. The uterine horns were carefully opened and each foetus together with its placentus placed into containers labelled, for analysis and stored at -70°C.

Fifteen sheep had eggs transferred to them, mostly 3 per sheep. Fourteen of the fifteen were pregnant at slaughter and of the 47 injected eggs transferred, 23 were represented by visually normal foetuses.

Example 5

Demonstration of Incorporation of FeLV Nucleic Acid

i) DNA extraction

High molecular weight DNA was prepared from 0.5-1.0g aliquots of placental or embryonic (brain and/or skeletal muscle) tissue by a method similar to that used for isolating such DNA from mouse tail tissue as described by Hogan et al. (1986).

Briefly the tissues were incubated overnight in 735 mcl of lysis buffer containing 50mM Tris pH8.0, 100 mM EDTA pH8.0, 0.5% SDA and 0.475 mg/ml proteinase K. The samples were removed to room temperature and gently extracted with 0.7 ml phenol (equilibrated with 1M Tris, pH8.0), followed by centrifigation (1200g, 3 mins, room temperature) to resolve the phases and careful removal of the aqueous (upper) phase. Two further extractions were carried out, using firstly a 1:1 (v/v) mixture of phenol and chloroform and secondly just chloroform. The nucleic acids were precipitated from the aqueous phase removed from the last extraction by the addition of 70 mcl 3M sodium acetate (pH 6.0) and 700 mcl 100% ethanol at room temperature. On mixing, the DNA formed a stringy precipitate which was immediately recovered by centrifugation (12000g, 0.5 minutes, room temperature). The supernatant was discarded and the pellet washed in 1 ml 85% ethanol in distilled water and recovered by centrifugation (12000g, 10 mins, room temperature). This washing and centrifugation process was repeated and the pellet then dried and resuspended in 100 mcl of 10 mM Tris, pH 7.6, 1 mM EDTA, 0.1 mg/ml RNase A (Sigma).

ii) Restriction enzyme digestion

20 mcl of DNA samples prepared as above (= 10-20 mcg of DNA) were digested to completion with the restriction enzymes KpnI or BamHI (Boehringer Corporation, Lewes, East Sussex). Digestions were carried out in the presence of 4 mM spermidine (Sigma) in appropriate buffer solutions at 37°C for 20 hours in the presence of 50 units of enzyme activity followed by the addition of a further 25 units of enzyme and digestion for another 8 hours.

iii) Agarose gel electrophoresis

DNA samples were electrophoresed in 0.8% agarose (Bethesda Research Laboratories) horizontal slab gels at 2.25 V/cm for 14.5 hours using a Tris/boric acid/EDTA buffer (0.089M Tris, 0.089M boric acid, 0.002M EDTA).

After electrophoresis, gels were stained in a solution of 0.5 mcg/ml ethidium bromide in distilled water and the DNA visualised by viewing under ultraviolet light (wavelength = 260 nm).

iv) Southern transfer and hybridisation

This procedure was carried out as described in Maniatis et al. (1982).

Briefly, after electrophoresis, the DNA samples in the gels were subjected to depurination (by immersion and gentle agitation in 0.25M hydrochloric acid, room temperature, 2 x 15 mins), followed by denaturation (immersion and gently agitation in 1.5M NaCl, 0.5M NaOH, room temperature, 2 x 30 mins) and lastly by neutralisation (immersion and gently agitation in 1M Tris, pH 8.0, 1.5M NaCl, room temperature, 2 x 30 mins). DNA was transferred to HYBOND-N nylon filters (Amersham Int. plc, U.K.) by capillary blotting for 20-24 hours. (The name 'HYBOND-N' is a trade mark). DNA was fixed to the filters by baking at 80°C in a non-vacuum oven for 2-2.5 hours.

Filters were prehybridized for 20-24 hours at 42°C with gentle agitation in a solution containing 50% formamide, 5 x Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS), 50mM Tris, pH 7.4, 10mM EDTA, 10% dextran sulphate, 250 mcg/ml denatured salmon sperm DNA and 3 x SSC. 100 x Denhardt's solution is 2% w/v bovine serum albumin, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone. 1 x SSC is 0.15M NaCl, 0.015M Na_3 citrate).

Radiolabelled probe DNA was prepared by a random priming method using the MULTIPRIME DNA labelling system and alpha ^{32}P'dCTP, specific activity 3000Ci/m.mol) (both obtained from Amersham Int. plc). (The word MULTIPRIME is a trade mark). The probe used was the FeLV specific U_3 probe shown in Figure 4b. This is a derivative of the plasmid pCT8 (Neil et al., 1984)

obtained by deletion of all the proviral sequences between the 2 KpnI sites one in each of the LTRs. Thus, this plasmid consists of a single FeLV LTR with flanking chromosomal DNA sequences in the plasmid pAT153. In Figure 3b, the positions of the sites for the restriction enzymes KpnI and HpaI are indicated. The solid black line between these two sites indicates the fragment of DNA used as a U_3-specific probe (600 bp).

Labelled probe DNA was added at a ratio of 1 x 10^6 cpm per ml of hybridisation fluid and the incubation continued for a further 16-18 hours at 42°C with gently agitation.

Filters were washed as follows: firstly in 2 x SSC, 0.1% SDS, room temperature, 2 x 10 mins, then in 0.5 x SSC, 0.1% SDS, 65°C, 2 x 60 mins.

Filters were dried to dampness, wrapped in SARAN-WRAP cling film and subjected to autoradiography with Dupont CRONEX intensifying screens at -70°C. (The words SARAN-WRAP and CRONEX are trade marks).

Figure 4 shows a Southern blot analysis of embryonic (E) and placental (P) DNA isolated from sheep embryos micro-injected subzonally with FeLV-B.

No HindIII digest of AK805 placental DNA was carried out due to a shortage of material. A and B represent different tissue samples from the AK805 embryo.

Sizes of bands were calculated by comparing the autoradiograph with a photograph of the gel taken after staining with a ruler placed alongside the gel. A total of 19 injected embryos were screened, of which two proved to be transgenic.

v) Results of Southern Blot Hybridisation

It can be seen from Figures 3a and 3b that the restriction enzyme digestion and probing strategy used in these studies should generate the following hybridising bands per single copy FeLV proviral integration event: a constant 1.2kb internal KpnI fragment and one other variable KpnI fragment > 0.54 kb; two variable HindIII fragments, one > 1.3 kb. and the other > 5.1 kb.

By examining the autoradiograph produced, it was clear that,

firstly, the U_3 probe used does not hybridise to any bands in the control tracks (DNA from uninjected embryo and placenta). Secondly, in the samples isolated from embryo and placenta 146, there are the following hybridising bands: KpnI - two at 1.2 kb, one each at 3.7 and 6.5 kb, HindIII - 1.9 kb, 3.6 kb and 2 bands at 6.0 kb, and in the samples isolated from embryo and placenta AK805, there are the following hybridising bands: KpnI - two at 1.2 kb, one each at 4.4 kb and 7.0 kb; HindIII - 4.3 kb, 2 bands at 10.0 kb and one at 18.5 kb. (Doublet bands were identified by the greater relative intensities). These data show that in each of the embryos there have been two independent integration events of an FeLV provirus into the embryonic genome and that these proviruses are for the greater part intact. The fact that the hybridisation patterns are the same in both embryonic and placental tissues strongly suggest that these events occurred before blastomeres became committed to either an embryonic or a trophoblastic lineage (i.e. probably at least before the 8-16 cell stage of embryonic development in the sheep - (Solter, 1987). Thus 2/19 (10.53%) embryos injected subzonally into the perivitelline space with FeLV-B incorporated FeLV-B DNA sequences into their genomes.

vi) Results of Microinjection

Forty-seven embryos injected in the perivitelline space with concentrated FeLV-B were transplanted into the oviducts of 15 synchronised recipient sheep. The embryos were removed at 6-7 weeks of gestation and twenty-three embryos were present. Nineteen of these embryos were analysed for FeLV-B proviruses by Southern blot hybridisation with an FeLV specific U3 region DNA probe. Two of these 19 embryos contained FeLV sequences and each contained 2 proviruses.

These results indicate that retroviral infection of ovine embryos is approximately 10-fold more efficient at producing transgenic ovine embryos when compared to a pooled published data on microinjection (Table 1).

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CLAIMS

1. A retroviral vector comprising:

(1) gag and pol gene products of a retrovirus;

(2) the env gene product of feline leukaemia virus sub-group B (FeLV-B); and

(3) retroviral sequences required for reverse transcription of retroviral RNA into DNA, for packaging, and, where integration of the transcribed DNA into the chromosome of a cell is required, also for integration, said retroviral sequences further containing foreign nucleic acid.

2. A vector according to claim 1 wherein the gag and pol gene products are of a feline leukaemia virus.

3. A cell culture containing or capable of releasing particles of a retroviral vector claimed in claim 1 or 2.

4. A cell culture according to claim 3 in the form of a retroviral packaging cell line containing nucleic acid coding for retroviral packaging proteins which comprise an env gene product of FeLV-B, and into which cell line a vector construct containing a packaging sequence and the foreign nucleic acid has been introduced.

5. A cell line according to claim 4 wherein a self-inactivating vector construct containing foreign nucleic acid has been introduced into the packaging cell line.

6. A cell line according to claim 5 wherein the self-inactivating vector construct is of DNA and comprises retroviral long terminal repeat (LTR) sequences, a packaging sequence and foreign DNA, wherein enhancer and promoter sequences in the 3'-LTR have been deleted or disabled.

7. A retroviral packaging cell line containing nucleic acid coding for retroviral packaging proteins which comprise an env gene product of FeLV-B.

8. A process for the production of a transgenic sheep or pig, the process comprising introducing a retroviral vector claimed in claim 1 or 2 or a retroviral venucleoprotein complex containing the DNA of a said retroviral vector intracellularly into a sheep or pig embryo.

9. A process according to claim 8 wherein a cell culture or line claimed in claim 3, 4, 5 or 6 is introduced into the embryo.

10. A transgenic sheep or pig produced by a process claimed in claim 8 or 9.

11. A process of introducing nucleic acid into an animal embryo, the process comprising injecting a viral vector or vector-releasing cell line through the zona pellucida into the perivitelline space.

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